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Anaerobic co-digestion of solid slaughterhouse wastes with agro-residues: Synergistic and antagonistic interactions determined in batch digestion assays

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Abstract

Different mixture ratios of solid cattle slaughterhouse wastes (SB), manure (M), various crops (VC), and municipal solid wastes (MSW) were investigated for biogas production. The objective was to explore possible significant synergistic effects obtained from the combination of these different substrates. The performance of the process was assessed in thermophilic anaerobic batch co-digestion assays, using a four factor mixture design and methane yield (Y_{CH_4}) and specific methane production rate (r_{sCH_4}) as response variables.

The highest methane yield, 655 NmL CH₄/g VS was obtained when equal parts (ww) of SB, M, VC, and MSW were combined, while the combination of SB, M, and MSW resulted in the highest specific methane production rate (43 NmL CH₄/g VS/d). A mixture design model was fitted to data in order to appraise synergistic and antagonistic interactions. Mixing all four substrates resulted in a 31% increase of the expected yield which was calculated from the methane potential of the individual fractions, clearly demonstrating a synergistic effect due to more balanced nutrient composition enhancing the anaerobic digestion process. However, no significant antagonistic effects were observed. In order to maximize both response variables simultaneously, a response surface method was employed to establish the optimal combination of substrate mixtures. The statistical results and analysis of the biological process gave a coherent picture of the results.

Keywords: anaerobic co-digestion; agro-industrial solid wastes; mixture experimental design; slaughterhouse wastes

1. Introduction

Due to the growing demand of meat in the world, the amount of organic solid wastes from meat producing industries is increasing every day. Approximately, between 20 – 50 % of the weight of the animal is not suitable for human consumption [1] and according to the Food and Agriculture Organization of the United Nations (FAO), the bovine meat production reached 65 million tons during the year 2011 [2]. Organic solid wastes from meat producing industries as well as from agricultural activities are considered suitable substrates for biogas production via anaerobic digestion (AD).

Because of their high content of fat and protein, slaughterhouse wastes are attractive substrates for biogas production, holding high methane yield potential. However, the process is sensitive and prone to failure [3, 4]. Degradation of long chain fatty acids (LCFA) has proved to be the limiting step in the hydrolysis process, and their accumulation results in toxicity problems for acetogenic bacteria and methanogenic archaea [5, 6]. Moreover, the residues often have low alkalinity [7], and are inclined to form floating scum during the digestion process [8]. Co-digestion of slaughterhouse wastes with other residues from agro-industrial activities has been proposed as a solution to these problems. The co-digestion process facilitates acquiring a better nutritional balance, and decreases the probability of ammonia and lipids inhibiting the process. Furthermore, the coexistence of different types of residues in the same geographic area enables integrated management, offering considerable environmental benefits, *e.g.* energy savings, recycling of nutrients within agriculture, and reduction of CO₂ emissions [9]. Another advantage is that, due to their different characteristics, co-digestion of these residues may enhance the performance of the anaerobic digestion process.

Several combinations of wastes are viable for co-digestion of slaughterhouse wastes.

Slaughterhouse wastes (mainly poultry and pig) have previously been co-digested with the organic fraction of municipal solid waste [8], fruit and vegetables wastes [10, 11], pig manure [11], food wastes [12], and sewage sludge [13, 14]. Synergistic effects occurring in the co-digestion process increase the gas yield from the substrates [15]. Pagés-Díaz et al. [16] studied co-digestion of solid cattle slaughterhouse wastes with different agricultural residues, and reported up to 43% higher methane yields than the expected methane yields of different mixtures, which were attributed to synergistic effects because of better nutritional balance for the microorganisms.

In most of these studies the co-digestion process involved two or three components, and the mixture composition was randomly selected. The composition of substrate determines the efficacy of the microbiological population [17], which in turn largely influences the biogas yield, the long term process stability, and the degradation rate of the solids.

Mixture experimental design is a useful technique to evaluate the interaction between the components of a blend, for maximizing the response. The design permits not only a determination of the linear blending parameter of each substrate, but also the second and third order of synergistic or antagonistic effects among the substrates. The independent factors are proportions of different components of a blend, and the principal goal of the design is to model the blending surface in order to predict the response of any mixture or combination of components [18].

Two recent studies were found applying an experimental design for evaluating the mixture ratios for co-digestion processes [19] [20]. Rao and Baral [19] investigated the co-digestion of sewage sludge with cow dung and garden wastes, or with cow dung and fruit juice wastewater, while Wang et al. [20] performed co-digestion of a mixture of animal manures (dairy, chicken and swine) with rice straw. In these studies at maximum of three components were asses in the batch assays and no deeper explanation was undertaken to fully understand

the synergistic effects in mixed substrates.

Biogas plants utilizing slaughterhouse waste are operating today below their full capacity [21]. Hence there is a need to improve the performance of these digesters. However, more information is required about how complex substrates perform together with slaughterhouse waste during co-digestion to achieve this goal. It is evidently necessary to assess the performance of the co-digestion process by an experimental evaluation, using a four factor mixture design to estimate a mathematical model and an explanation of the results in terms of biological process. Hence, the strategy in the present study was to apply a four factor mixture design for the evaluation of all two and three factor synergistic and antagonistic interactions in the co-digestion process, and to discuss the biological influence of different substrate compositions on the anaerobic co-digestion of solid cattle slaughterhouse wastes mixed with three different residues from agricultural activities.

2. Materials and Methods

2.1 Raw Materials

Four different raw materials, solid cattle slaughterhouse wastes (SB), manure (M), various crops (VC), and the organic fraction of municipal solid waste (MSW) were used for the anaerobic co-digestion experiments. SB (*i.e.*: cow rumen, blood, and gut fill components) was provided by a biogas plant in Kristianstad (Sweden). A mixture of 50% ww pig manure, 25% ww horse manure and 25% ww cow manure (all obtained from a farm outside Borås, Sweden) was prepared and subsequently used as a fraction of manure. The composition of the manure fraction was determined based on the data from four different slaughterhouse enterprises in Cuba taking into account the amount of different kinds manure generated by the animals found at the farms nearby. The various crop substrates were prepared by mixing 70% ww of green fruit and vegetables residues with 30% ww of straw. Similarly, this composition was

determined by considering that it is always less amount of straw available, then green fruit and vegetable waste remaining after the harvest in Cuba. Municipal solid waste was obtained from a large-scale biogas plant (Borås Energy & Environment AB, Borås, Sweden). Every individual fraction was cut and minced using an electric blender (Blender HGB55E, Commercial Torrington, USA). These four substrate fractions were then used for the preparation of the different mixtures (M_i). After that the prepared mixtures were stored at -18°C until further investigation.

2.2 Experimental design

The experimental design of the present study comprised a four-factor simplex-centroid mixture design, consisting of pure fractions and mixtures of two, three, and four components at wet weight ratios (% ww) according to Table 1. The design experiments were replicated three times. Response variables were methane yield (Y_{CH_4}) and specific methane production rate (r_{sCH_4}). This design allows estimation of a cubic polynomial model, where first order coefficients measure the relative effect of pure substrates, while quadratic and cubic terms measure the synergistic or antagonistic interactions between the fractions.

A linear mixing model without any synergistic or antagonistic interactions may be expected to produce a methane yield proportional to the amount of VS (volatile solids) supplied by the individual fractions. The experimental results were accordingly analyzed by fitting a model that expresses the responses as functions of the VS content of the components in the respective mixtures. The fractions were thus characterized as the VS content in relation to the resulting contribution %VS of total VS in the calculated mixtures (Table 1).

2.3 Anaerobic batch digestion assays

The anaerobic batch experiment was performed in accordance with a method described by Hansen et al. [22]. The assays were carried out under thermophilic conditions ($55^{\circ}\text{C} \pm 1^{\circ}\text{C}$) for 70 days, using 2 L glass bottles as reactors. A large-scale biogas plant, operating at thermophilic conditions (Borås Energi & Miljö AB, Borås, Sweden), provided an inoculum holding a content of 3.8% TS (total solids) and 2.1% VS. To remove large and undigested particles the inoculum was filtered through a 2 mm porosity sieve, after which it was allowed to stabilize stored in an incubator at 55°C for three days. Each bottle was fed with an appropriate amount of a substrate mixture (M_i) and 400 mL inoculum, keeping a VS ratio ($\text{VS}_{\text{substrate}}$ to $\text{VS}_{\text{inoculum}}$) at 1:2 in all setups. In order to reproduce the real conditions in a full scale plant no micro and macronutrients were added. Blank experiments, containing 400 mL inoculum, disclosed any methane production of the inoculum itself. Furthermore, control assays were also performed with pure cellulose (Cellulose Fibrous Long, Sigma Aldrich, Germany) as substrate to ensure the quality of the inoculum. The bottles were subsequently sealed with rubber septa and aluminum caps (Apodan Nordic, Copenhagen, Denmark). The reactors were flushed with a gas mixture comprising 80% N_2 and 20% CO_2 for 3 min, ensuring anaerobic conditions. Each experimental setup was prepared in triplicate. The bottles were shaken once a day during the incubation period of 70 days.

Gas was regularly sampled from the headspaces of each bottle, using a 250 μL pressure-tight syringe (VICI, Precision Sampling Inc., Baton Rouge, LA, USA), and the samples were immediately analyzed by gas chromatography. To avoid build-up of high pressure in the reactors, excess gas was released after each analysis through a needle (Sterican[®] Ø 0.4x20 mm, B. Braun, Germany), after which the gas analysis was repeated. Assuming ideal gas mixtures and using the ideal gas law, the methane content in the head space can be calculated using the data from the GC measurements and without measuring the actual pressure in the bottles [22]. The amount of CH_4 produced between two subsequent sampling in the head

space of each flask was calculated as described previously by Teghammar et al. [23]. A gas with known composition was used as standard in each measurement occasion.

As response variables, methane yield (Y_{CH_4}) and specific methane production rate (r_{sCH_4}) were used in order to evaluate the performance of the anaerobic co-digestion process. Both responses were determined using the methods published previously by Pagés-Díaz et al. [24]. Furthermore, the time (t_{Grenz}) during which methane production of two consecutive points differed less than 1% was also calculated [24].

2.4 Analytical methods

Total solids (TS) and volatile solids (VS) were analyzed in accordance with Sluiter et al. [25]. TS were determined after heating at 105°C to a constant weight, and VS by incinerating the dried samples for 4h in a muffle furnace at 575°C. The pH of the solid materials was, in accordance with the VDI 4630 guidelines, analyzed by LUFA Bd. III 8.1 [26]. Total Kjeldahl nitrogen (TKN) was measured by the semi-micro-Kjeldahl method, following a Swedish standard method, SS-EN 25663/NMKL 6-3 [27]. Total protein content was calculated from the TKN content, using a conversion factor of 6.25 (for meat) in accordance with Gunaseelan [28]. Ammonium nitrogen ($NH_4^+ - N$) was determined with a Swedish standard method, SIS 028131-1 [29], and the amount of organic nitrogen was estimated by calculating the difference between TKN and $NH_4^+ - N$. Lipid quantity was measured by the method NMKL 131 [30], and the remaining organic content was assumed to be carbohydrates.

The composition of the produced biogas was determined by gas chromatography. The gas chromatograph (Auto System Perkin Elmer, USA) was equipped with a packed column (Perkin Elmer, 6' x 1.8" OD, 80/100, Mesh, USA) and a thermal conductivity detector (Perkin Elmer, USA). An inject temperature of 150°C was applied, and the carrier gas was nitrogen operated with a flow rate of 20 mL/min at 75°C. Data were processed assuming the ideal gas

law in accordance with Hansen et al. [22]. All methane volumes are presented at normal conditions (0 °C, 101.325 kPa) according to ISO 10780 [31].

2.5 Statistical analyses

The effect of quaternary mixture compositions of SB, M, VC, and MSW substrates on methane yields (Y_{CH4}) and on specific methane production rates (r_{sCH4}) was investigated by means of a simplex centroid design. The combinations chosen were the pure blend, binary blends, tertiary blends and the overall centroid. The design has $2^q - 1$ points, where $q=4$ represents the numbers of components in the system and x_i represents the proportion of i^{th} constituent in the mixture. The proportion of each component varied between 0 and 1 according to Eq. (1):

$$\sum_{i=1}^4 x_i = x_1 + x_2 + x_3 + x_4 = 1; \quad 0 \leq x_i \leq 1 \quad \text{Eq. (1)}$$

The experimental mixture ratios for a quaternary system are listed in Table 1. The setup of the experimental design was based on wet weight proportions. However, in order to be able to interpret the high order terms as synergistic/antagonistic effects the model was analyzed with mixtures expressed in terms of volatile solid ratios within the mixtures (Table 1).

In order to predict the response variable ($\hat{Y}$) as a function of the four components, polynomial models were employed to link the response with the composition of the mixture. A special cubic model with 14 coefficients was used for the 4-component mixture design:

$$\hat{Y} = \sum_{1 \leq i \leq 4} \beta_i x_i + \sum_{1 \leq i < j \leq 4} \beta_{ij} x_i x_j + \sum_{1 \leq i < j < k \leq 4} \beta_{ijk} x_i x_j x_k \quad \text{Eq. (2)}$$

The first sum represents the estimated linear mixing; the quadratic part with coefficients β_{ij} represents the synergistic or antagonistic blending effects between components pairs, and the cubic part denotes the three component interactions.

All three models (linear, quadratic, and cubic) were fitted for each response variable by means of regression methods, and analyzed with ANOVA. The significance of the regression models was evaluated at 5% significance level ($p\text{-values} \leq 0.05$), and pooled standard deviations were used to assess the size of experimental errors. The hypothesis that the selected model is adequate to describe the experimental data ($p\text{-values} \geq 0.05$) was verified by the lack of fit test. MINITAB® Release 14.12.0 software package (Minitab Inc., State College, PA, USA) was used for the analyses of the results.

3. Results and Discussion

3.1 Mixture characterization

The main characteristics of individual substrates and mixtures are summarized in Table 2. The investigated slaughterhouse and agro-industrial wastes mixtures are substrates with high organic matter content showing similar characteristics as in previous reports [8, 10, 32, 33].

SB showed the highest lipid and protein content (175 g/kg_{FM} and 130 g/kg_{FM}, respectively) among the substrates examined in the present investigation, making it an attractive substrate for biogas production. Nevertheless, as reported by other studies [8, 10, 12], the high amounts of lipids and proteins in slaughterhouse waste may cause inhibition. Consequently, this waste is not optimal to use alone as substrate for biogas production [17]. High lipid content may result in slower methane production and lower hydrolysis rate [34]. The other individual substrates studied (M2, M3 and M4) had lower proportions of lipids and proteins, but higher contents of carbohydrates (77 – 287 g/kg_{FM}). Hence, co-digestion of these substrates with slaughterhouse wastes would, thanks to complementary characteristics, lead to a better,

balanced composition.

The organic nitrogen content of SB was high (20 g N/kg_{FM}) in comparison with the rest of the individual fractions (3 – 4 g N/kg_{FM}). However, the TKN (0.16 – 0.89 g N/L) which corresponds to both inorganic and organic nitrogen together, did not, in any of the reactors, reach the inhibitory level of 4 g/L reported for ammonia nitrogen alone by Angelidaki and Ahring [35] who determined this value under similar conditions as those used in the present study, *i.e.* 55°C and pH around 8.

3.2 Model fitting and regression analysis

A quaternary mixture of different agro-residuals, such as SB, M, VC and MSW were (as described above) subjected to anaerobic co-digestion. Table 3 summarizes predicted as well as experimental values obtained in terms of Y_{CH_4} and r_{sCH_4} . Linear, quadratic and special cubic models were used to obtain least square fits of both response variables and Table 4 shows the results of the analysis of variance (ANOVA) and regression significance of the models, displaying the sum of squares attributable to both model and error.

The special cubic model proved to be the best suited for both response variables (Table 4). The p-values for Y_{CH_4} as well as r_{sCH_4} were higher than 0.05 (0.4197 and 0.9836, respectively) in the lack of fit test for this model. Estimated regression coefficients, standard errors, and p-values for the special cubic model are listed in Table 5.

The significance of regression coefficients verified that coefficients corresponding to M5, M10, M11, M12, and M13 had significant effects on methane yield, while coefficients corresponding to M5, M10, M6, and M12 had significant effects on specific methane production rate (Table 5). The fitted models for the response variables can be expressed by equations 3 and 4 below:

$$\begin{aligned}\hat{Y}_{CH_4} = & 608 x_{SB} + 384 x_M + 422 x_{VC} + 534 x_{MSW} + 379 x_{SB}x_M + 296 x_{SB}x_{MSW} \\ & + 3380 x_{SB}x_Mx_{VC} + 2588 x_{SB}x_{VC}x_{MSW} + 2045 x_Mx_{VC}x_{MSW}\end{aligned}\quad \text{Eq. (3)}$$

$$\begin{aligned}\hat{r}_{sCH_4} = & 13 x_{SB} + 30 x_M + 37 x_{VC} + 45 x_{MSW} + 76 x_{SB}x_M + 54 x_{SB}x_{MSW} \\ & - 41 x_Mx_{VC} + 471 x_Mx_{VC}x_{MSW}\end{aligned}\quad \text{Eq. (4)}$$

3.3 Analyses of response variables

The responses variables (experimental and predicted) evaluated during the experiments are presented in Table 3. Figure 1 shows cumulative methane production and specific methane production rates obtained for the different mixtures. Furthermore, a surface response representation, displayed as a function of the mixture ratios of the four substrates, is shown in Figure 2, each of these graphs providing a plot of the predicted response as a function of any of the experimental components. The height of the surface represents the predicted value, which is plotted over the range of the experimental components [18]. These plots are useful for establishing desirable response values and mixture compositions.

Among the individual fractions, SB, with its high content of fats and proteins, produced a higher Y_{CH_4} but a lower r_{sCH_4} compared to the other individual fractions (M2, M3, M4) (Figure 1). This characteristic profile has previously been reported by Broughton et al. [5], Cuertos et al. [8], Pagés-Díaz et al. [16], and Palatsi et al. [17]. From the plots of Y_{CH_4} (Figure 2A), it was evident that the zones of high yield were located towards the side of the triangle, having SB and MSW as vertices. These substrate combinations were expected to have a positive effect on methane yield from the analyses of ternary and quaternary mixtures. On the other hand, M and VC were the individual fractions contributing least to methane yield. However, presence of these substrates in the mixture combinations does play an important role, as they help balancing the macro- and micronutrients of the system, improving the feed composition,

and contributing to the buffer capacity of the system. Thus, they mainly support the stability of long-term processes. This was confirmed in the co-digestion comprising all four substrate fractions (M15), which produced the highest methane yield of 655 NmL CH₄/g VS (Table 3). In the present study, co-digestion of different substrate mixtures generally resulted in methane yields ranging from 434 to 655 NmL CH₄/g VS. These yield results are in agreement with previous research by Cuetos et al. [8] and Bayr et al. [32]. Although the operating conditions were not identical in those studies, methane yields from similar wastes ranged from 400 to 700 NmL CH₄/g VS.

Evaluating the other response variable, *i.e.* r_{sCH_4} , the tridimensional surface plots (Figure 2B) showed that the zones of maximum response were situated towards the side of the triangle, having VC and MSW as vertices. Nevertheless, the optimal result was obtained by combining the three substrate fractions M, VC, and MSW (M12), which resulted in a r_{sCH_4} of 43 NmL CH₄/g VS/d. Furthermore, the calculated t_{Grenz} values (see Table 3), which can be used for defining possible hydraulic retention times (HRT) in semi-continuous operations, revealed that SB alone are still producing biogas after more than 40 days with the lowest r_{sCH_4} of 13 NmL CH₄/g VS/d. The other individual substrates (M2 – M4), as well as the different mixtures (M5 – M15), had an average t_{Grenz} value of 26 days. The t_{Grenz} should be considered when designing continuous operations, and when determining the size of a reactor. However, t_{Grenz} is also dependent on the quality of the inoculum and its capability of adapting to the substrate.

3.4 Synergistic and antagonistic interactions

Simultaneous presence of several types of residues in the co-digestion process may, due to significant synergistic interactions, improve the process [10, 16, 36], producing a higher methane yield than is obtained from individual fractions. Many of the catalytic centers of the

enzymes involved in methanogenic pathways are micro nutrients dependent [37]. A mixture of different substrate fractions with different characteristics can provide all of the nutrients and trace elements the microorganisms need. This heterogeneity in the substrate composition will in turn lead to that several more different types of microorganisms can growth in the digester stimulating the anaerobic degradation process.

In the present work, it was possible to model the expected methane yield assuming no synergistic effects using the linear part of the special cubic model. The effects of two- and three-factor (substrate) interactions were included in the remaining part of the model (quadratic and cubic terms), allowing the model to predict synergistic and antagonistic interactions. These correspond with the obtained methane yields presented in Table 3 and Figure 3.

From the fitted model we can deduce the individual performance of pure substrates. SB had the highest coefficient for Y_{CH_4} and the lowest coefficient for r_{sCH_4} . Mixtures corresponding to M5, M10, M11, M12 and M13 had significant synergistic effects (p-values ≤ 0.05) on methane yield. The ternary mixture of M11 had the highest beta coefficient (3380), exerting a strong synergistic interaction (p-value = 0.000). M6 and M7 were assumed to be synergistic, but showed no significant interaction (p-value > 0.05). On the other hand, the binary blends, *i.e.* M8 and M9, exhibited antagonistic interaction, although not reaching significance (Table 5). These results are in agreement with the size of synergistic and antagonistic effects displayed in Figure 3, with the exception of M14 (SB + M + MSW) that showed a significant synergistic effect even though, according to the model, its beta coefficient was not significant (Table 5 and Figure 3). This can be explained by the fact that in the model, the increase over the linear mixing was not solely stemming from the ternary term (SB + M + MSW), but also from the binary terms (SB + M, SB+ MSW, M + MSW). The combinations of SB + M and SB + MSW are significant and contribute synergistically to the effects obtained for M14 even

though when the combination of M + MSW had no significant effect. The largest increase in Y_{CH_4} (in comparison with the expected value) was 31%, and was obtained from the quaternary mixture (M15) followed by M11, achieving an enhancement in Y_{CH_4} by 26% (Figure 3). M6 and M7 brought about only a slight increment (7% and 1%, respectively) and displayed no significant interactions (Figure 3).

The specific methane production rates evolved in a similar pattern to methane yields in terms of synergistic and antagonistic interactions. The mixtures M5, M10, and M12 displayed significant synergistic interactions (p-values ≤ 0.05). Consequently, a combination of these substrates, *i.e.* SB + M, SB + MSW, and M + VC + MSW, would be a good strategy to implement in order to attain high methane yields in conjunction with high degradation rates in large scale biogas plants when applying HRTs for about 26 days (t_{Grenz}). The mixture of M6 displayed significant antagonistic effects as judged by a negative beta coefficient value (- 41) and a p-value of 0.043 (Table 5). This is due to the presence of VC fraction in major quantities in the VS content of the mixture (Table 1). This substrate had the lowest pH value (4.16), which most likely would lead to acidification in a semi-continuous operation. The high biodegradability of the fruit and vegetable waste fraction results in a rapid production of volatile fatty acids [38].

The three factor interactions (M11, M12, M13, M14 and M15) showed an increment comparing to the two factor interactions (M5, M6, M7, M8, M9 and M10) for both response variables. In average, the ternary mixture combinations brought about an increase of methane yield with 15%, while specific methane production rate averagely increased by 18% (see results presented in Table 5 and Figure 3), in comparison with the binary mixture ratios.

Finally, we can conclude, that the co-digestion process proved to be a good option for treating this waste fraction. Correlating synergistic and antagonistic interactions brought about from the biological process with statistical results was found to be feasible. Hence, statistical

analysis may be considered a valuable tool for evaluation of biological processes, such as anaerobic digestion.

3.5 Optimization of the response variables

Experimental mixture designs are classically applied as efficient means to predict or to optimize a response variable Y , resulting from the mixture of several components. Response optimization of mixture proportions incorporates the selection of an optimal component combination from a set of available alternatives. The objective of the optimization process is to find a combination of substrates that maximizes the methane yield and the specific methane production rate in conjunction. Since the two responses variables have different optimal mixtures, a simultaneous optimization was done using the composite desirability as a function that weights the methane yield and the specific methane production rate against each other.

The optimum mixture ratio computed from the analysis was that 35.41% of VS from SB, 0.46% of VS from VC and 64.13% of VS from MSW, resulting in 636.6 NmL CH₄/g VS methane yield, with a specific methane production rate of 45.6 NmL CH₄/g VS/d (Figure 4). The composite desirability turned out to be 0.94, implying that the optimization was appropriate. The findings were consistent with our previous observations that using MSW as a component in a major proportion produces a high methane yield along with a high specific methane production rate.

4.

Co

conclusions

In this study, the co-digestion of solid cattle slaughterhouse wastes with different residues from agro-industrial activities was investigated by using a mixture experimental design. It was possible to relate the synergistic and antagonistic interaction obtained from the biological

process to the statistical results. Methane yields obtained were higher by up to 31% compared to the values expected from the linear mixing model, due to significant synergistic effects in some mixture combinations. In general, it was possible to correlate biological behavior and statistical results; hence statistical analysis is a valuable tool to evaluate biological processes, as anaerobic digestion.

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TABLE LEGENDS

Table 1. Mixture compositions, following simplex centroid design, and the experimental setups for the batch co-digestion assays

Table 2. Chemical characterization of each substrate mixture (M_i) investigated in anaerobic batch digestion assays.

Table 3. Experimentally determined and predicted response variables (Y_{CH_4} and r_{sCH_4}) obtained for the different substrate mixtures, along with Grenz time (t_{Grenz}) and pH

Table 4. ANOVA for the regression results, using Y_{CH_4} and r_{sCH_4} as response variables

Table 5. Estimated regression coefficients, standard errors for the estimates and statistical significance obtained by the fitted special cubic model for both response variables (Y_{CH_4} and r_{sCH_4})

FIGURE LEGENDS

Figure 1. Cumulative methane yield (A) and specific methane production rate (B) corresponding to different waste mixture compositions during the batch co-digestion assays

Figure 2. Three-dimensional mixture surface plots for methane yield (A) and specific methane production rate (B)

Figure 3. Synergistic and antagonistic effects during the experimental co-digestion of different mixture ratios. White bars correspond to methane yields obtained by the first linear part of the special cubic model (with no synergistic and antagonistic interactions), while grey bars correspond to methane yields obtained by the full special cubic model (taking into account synergistic and antagonistic interactions)

Figure 4. Optimization solution and overlaid contour plot for methane yield and specific methane rate

Table 1.

Mixtures	Substrate [*] (g)	Mixing ratios							
		SB		M		VC		MSW	
		% of ww_{total}	% of VS_{total}	% of ww_{total}	% of VS_{total}	% of ww_{total}	% of VS_{total}	% of ww_{total}	% of VS_{total}
M1	17	100	100	0	0	0	0	0	0
M2	30	0	0	100	100	0	0	0	0
M3	19	0	0	0	0	100	100	0	0
M4	27	0	0	0	0	0	0	100	100
M5	20	50	64	50	36	0	0	0	0
M8	18	50	53	0	0	50	47	0	0
M10	21	50	62	0	0	0	0	50	38
M6	22	0	0	50	39	50	61	0	0
M9	25	0	0	50	48	0	0	50	52
M7	23	0	0	0	0	50	58	50	42
M11	20	33.3	41	33.3	23	33.3	36	0	0
M14	22	33.3	45	33.3	26	0	0	33.3	29
M13	21	33.3	40	0	0	33.3	35	33.3	25
M12	23	0	0	33.3	27	33.3	43	33.3	30
M15	21	25	32	25	18	25	29	25	21

^{*} Amounts of individual substrate mixture added in each batch

% of ww_{total} : shows how much percentage each individual fraction counts up within the wet weight (ww) of the mixture, M_i

% of VS_{total} : shows how much percentage each individual fraction counts up within the volatile solid (VS) content of the mixture, M_i

Table 2.

Mixtures		M1	M2	M3	M4	M5	M6	M7	M8
pH		5.87	8.36	4.16	5.21	6.14	4.92	4.64	4.79
Total solids	%	26	35	24	18	31	30	21	25
Volatile solids	% _{TS}	95	40	90	84	67	65	87	93
Kjeldahl – N	g	21	4	3	4	13	4	4	12
	N/kg _{FM}								
	g/L*	0.89	0.31	0.16	0.29	0.64	0.20	0.21	0.55
NH ₄ ⁺ – N	g/kg _{FM}	1.4	0.64	0.15	0.31	1.02	0.39	0.23	0.77
	g/L*	0.059	0.048	0.007	0.021	0.052	0.021	0.013	0.035
Organic – N	g/kg _{FM}	20	3	3	4	12	4	4	11
	g/L*	0.83	0.27	0.15	0.26	0.59	0.18	0.20	0.51
Lipids	g/kg _{FM}	175	4	2	12	90	3	7	89
Proteins	g/kg _{FM}	130	26	21	26	78	24	24	76
Carbohydrates	g/kg _{FM}	1	180	287	77	91	234	182	144
Mixtures		M9	M10	M11	M12	M13	M14	M15	
pH		5.31	5.51	4.99	5.03	4.83	5.66	4.92	
Total solids	%	27	22	28	26	23	26	26	
Volatile solids	% _{TS}	62	90	74	71	89	72	77	
Kjeldahl – N	g/kg _{FM}	4	13	9	4	9	10	8	
	g/L*	0.26	0.67	0.47	0.22	0.49	0.54	0.43	
NH ₄ ⁺ – N	g/kg _{FM}	0.47	0.85	0.72	0.36	0.61	0.77	0.63	
	g/L*	0.03	0.045	0.036	0.021	0.032	0.043	0.033	
Organic – N	g/kg _{FM}	4	12	8	4	8	9	7	
	g/L*	0.23	0.62	0.44	0.20	0.46	0.49	0.40	
Lipids	g/kg _{FM}	8	94	60	6	62	63	48	
Proteins	g/kg _{FM}	26	78	58	24	58	60	51	
Carbohydrates	g/kg _{FM}	129	39	154	180	120	85	136	

* Nitrogen content in the reactor

FM: Fresh matter

TS: Total Solid

Table 3.

Mixtures	Y_{CH_4} <i>Experimental</i> ^a (NmLCH ₄ /gVS)	$\hat{Y}_{CH_4}$ <i>Predicted</i> ^b (NmLCH ₄ /gVS)	r_{sCH_4} <i>Experimental</i> ^a (NmLCH ₄ /gVS/d)	$\hat{r}_{sCH_4}$ <i>Predicted</i> ^b (NmLCH ₄ /gVS/d)	t_{Grenz} (d)	pH
M1	609 (±48)	608 (±28)	13 (±4)	13 (±5)	45	8.43
M2	384 (±23)	384 (±23)	30 (±9)	30 (±4)	23	8.33
M3	422 (±22)	422 (±23)	35 (±6)	37 (±4)	21	8.30
M4	535 (±35)	534 (±23)	44 (±9)	45 (±4)	21	8.42
M5	613 (±55)	615 (±23)	37 (±9)	37 (±4)	27	8.48
M6	432 (±50)	434 (±23)	22 (±3)	24 (±4)	29	8.38
M7	470 (±3)	471 (±23)	31 (±6)	32 (±4)	25	8.34
M8	461 (±50)	464 (±22)	23 (±3)	23 (±4)	30	8.35
M9	461 (±53)	462 (±23)	31 (±3)	31 (±4)	25	8.33
M10	647 (±50)	650 (±28)	35 (±4)	38 (±5)	31	8.39
M11	622 (±41)	615 (±21)	34 (±3)	34 (±3)	29	8.40
M12	535 (±38)	531 (±22)	43 (±7)	43 (±4)	23	8.20
M13	621 (±51)	614 (±21)	34 (±6)	34 (±3)	29	8.38
M14	617 (±22)	612 (±22)	36 (±9)	38 (±4)	27	8.41
M15	641 (±20)	655 (±16)	40 (±5)	40 (±3)	26	8.25

^a Experimental mean values and standard errors from triplicate samples^b Predicted values and standard errors from the fitted model

Table 4.

Response variable	Sum of Squared	Standard Error	Degree of freedom	R-Squared (%)	p-value
Y_{CH4}					
Linear					
Regression	199907	68	3	53	0.0000
Lack of fit	136781		11		0.0000
Pure error	43984		28		
Quadratic					
Regression	283229	54	9	74	0.0000
Lack of fit	53459		5		0.0003
Pure error	43984		28		
Special Cubic					
Regression	335634	39	13	88	0.0000
Lack of fit	1054		1		0.4197
Pure error	43984		28		
r_{sCH4}					
Linear					
Regression	742	8	3	21	0.0033
Lack of fit	1638		11		0.0037
Pure error	1197		28		
Quadratic					
Regression	1795	7	9	50	0.0008
Lack of fit	585		5		0.0391
Pure error	1197		28		
Special Cubic					
Regression	2380	6	13	67	0.0006
Lack of fit	0.018		1		0.9836
Pure error	1197		28		

Table 5.

Coefficient	Mixtures	<i>Effect on</i> Y_{CH4}			<i>Effect on</i> r_{sCH4}		
			SE	p-value		SE	p-value
β_{SB}	M1	608	28	*	13	5	*
β_M	M2	384	23	*	30	4	*
β_{VC}	M3	422	23	*	37	4	*
β_{MSW}	M4	534	23	*	45	4	*
β_{SB*M}	M5	379	130	0.007 ^a	76	21	0.001 ^a
β_{SB*VC}	M8	-229	116	0.057	-3	19	0.864
β_{SB*MSW}	M10	296	143	0.047 ^a	54	23	0.028 ^a
β_{M*VC}	M6	111	118	0.352	-41	19	0.043 ^a
β_{M*MSW}	M9	-0.04	112	0.999	-28	18	0.140
β_{VC*MSW}	M7	9	114	0.939	-32	19	0.099
$\beta_{SB*M*VC}$	M11	3380	799	0.000 ^a	138	130	0.297
$\beta_{SB*M*MSW}$	M14	15	879	0.986	-77	143	0.597
$\beta_{SB*VC*MSW}$	M13	2588	771	0.002 ^a	83	126	0.516
$\beta_{M*VC*MSW}$	M12	2045	816	0.018 ^a	471	133	0.001 ^a

^a p - value ≤ 0.05 (95% CI) denoted significance

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Highlights

- Batch co-digestion of slaughterhouse wastes with three other residues was investigated.
- A four factor mixture design was used to find optimal mixture composition.
- Certain mixture ratios performed better due to significant synergistic effects.
- Equal amounts of SB:M:VC:MSW produced 31% more methane than expected.
- It was possible to correlate biological behavior and statistical results.